and Dohan examined the pancreatic remnants of rats that had had pronounced hyperglycemia for from 2 weeks to several months following partial pancreatectomy and found almost no damage.[21] In similarly treated animals hyperplasia instead of damage was reported by Friedman and Marble.[22] The same type of change was observed after the administration of anterior pituitary extract.[10] In order to produce glycosuria consistently in the rat with anterior pituitary extract, partial pancreatectomy is necessary,[23] although adrenal steroids cause temporary insulin resistant diabetes.[24] However, Peterson has recently reported complete degranulation of rat islet beta cells within 15 minutes of the intracardiac injection of 3.0 g of glucose/kg.[25] This lasts 48 hours and is associated with temporary impairment of glucose tolerance. Similar but less consistent degranulation was found after one intraperitoneal injection of glucose in the guinea pig by Gomori *et al.*[26]

In canine anterior pituitary diabetes, proliferation of the islet cells may precede the exhaustive or degenerative changes which accompany the onset of permanent diabetes.[27] Our observations indicate that the carbohydrate tolerance increased with each increase in feeding, glycosuria decreasing when the diet was maintained at a level which had initially caused moderate sugar excretion. This adaptation is evidently implemented by islet hyperplasia. Although it might be possible to prevent hyperplasia and produce islet damage by increasing the volume of food more rapidly this would probably be difficult without causing fatal "food-shock." It is conceivable that some relatively small additional stimulus, perhaps the administration of anterior pituitary extract or adrenal steroids, might have produced exhaustion of the islets of our rats. Older rats may be more susceptible.[28]

Summary. Degranulation of beta cells of the pancreatic islets and islet hyperplasia have been observed in rats which were force-fed large amounts of high carbohydrate, otherwise balanced diets for periods of as long as 8 weeks. Glycosuria and, in rare instances, ketonuria occurred.

[21] Lukens, F. D. W., and Dohan, F. C., *Endocrinol.*, 1942, **30**, 175.

[22] Friedman, N. B., and Marble, A., *Endocrinol.*, 1944, **29**, 577.

[23] Long, C. N. H., *Harvey Lect.*, 1936-37, **32**, 194.

[24] Ingle, D. J., Sheppard, R., Evans, J. S., and Kuizenga, M. H., *Endocrinol.*, 1945, **37**, 341.

[25] Peterson, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 352.

[26] Gomori, G., Friedman, N. B., and Caldwell, D. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 567.

[27] Richardson, K. C., and Young, F. G., *Lancet*, 1938, **1**, 1098.

[28] Martinez, C., *Rev. Asoc. med. argent.*, 1946, **60**, 666.

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17175. Inorganic Aging of the Plasma Layer of Tissue Cultures.

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The problem of maintaining mammalian cells *in vitro* for long intervals in an original or lightly patched plasma base may be broken down into two aspects: (a) the avoidance of calcification during the usual transfer intervals of two to four weeks and (b) the problem of an inorganic aging which complicates more prolonged maintenance. Though the control of calcification difficulties during the usual transfer intervals seems feasible by the measures summarized in the preceding paper,[1] solution of the long-term problems

[1] Hanks, J. H., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 328.

arising from inorganic aging appears more formidable.

It is a classical observation that even in the absence of evident calcification the plasma layer of cell cultures gradually becomes less and less favorable to cell growth. Simms and Stillman[2] have presented evidence that tryptic digestion removes from adult tissues and from the plasma of cell cultures an inhibitor having some of the properties of calcium phosphate. It is also known that inorganic gels undergo changes in viscosity, pH, extinction coefficients, and other physical properties during aging. These changes can be explained in part by preferential liberation and adsorption of ions, that is by ionic exchange.[3] Particularly in circumstances where a gel is in contact with supernatant fluids which are changed repeatedly, the concentration of preferentially adsorbed ions will become radically different from the ionic analysis at the moment the gel is formed.[4] Such procedures are an inherent feature of the long-term tissue culture technics.

It is the purpose of the present communication to present evidence concerning the adsorption of calcium by the plasma layer of tissue cultures and the difficulty of decalcifying this substrate. Methods have been summarized in the preceding paper.[1]

Experimental observations. The firmness with which calcium and phosphates are adsorbed to the plasma layer became apparent as the result of attempts to remove opacity from cell colonies and plasma which had become calcified. This opacity could not be diminished by using supernatant media prepared by diluting the serum and embryo juice in salt solution lacking both Ca and P or by prolonged depression of pH during cultivation within physiological limits of H ion concentration. Incorporation of 0.1 to 0.2% sodium citrate in media of minimal Ca and P levels or in mildly acidified washing solutions killed the cells without clearing the plasma. Direct extraction with N/100 HCl in the balanced salt solution or weaker acids in the balanced salt solution likewise failed to clear the plasma before the cells were irreparably damaged. The deposits were rapidly removed by N/10 HCl, which was fatal to cells. Optimal cell survival followed rapid clearing of the plasma in N/40 HCl in the salt solution for 5 to 20 minutes as required. Since the complicated procedure required to effect this result and obtain prompt neutralization without cell damage is not to be recommended,* these observations are summarized only in order to illustrate the fact that the Ca adsorbed by the plasma is not brought into solution by complex-forming anions such as citrate and is liberated only by ionic exchange with fairly high concentrations of H ion.

Other evidence of the role of ionic exchange in the inorganic aging of plasma was obtained from data on the increased deposition of Ca in the plasma layer during repeated renewal of cultures with supernatant fluids which do not produce calcification within the usual transfer intervals. After 6 weeks of cultivation in media composed of human serum 40% and beef embryo juice 4% diluted in the salt solution containing Ca 5 mg% and P 2.2 mg% (six renewals) the slightly opalescent plasma layers of 10 cultures were rinsed and extracted with N/10 HCl. Analysis revealed that this plasma contained from 24 to 40 mg% of Ca, *i.e.*, three to five times the original concentration of Ca in the plasma.

When the same formula (sera 40%, embryo

[2] Simms, H. S., and Stillman, N. P., *J. Gen. Physiol.*, 1937, **20**, 603.

[3] Hanks, J. H., and Weintraub, R. L., *J. Phys. Chem.*, 1937, **41**, 583.

[4] Jenny, H., *J. Phys. Chem.*, 1932, **36**, 2217.

* A more practical procedure is the complete removal of opaque plasma by digestion with 0.5% trypsin or pancreatin in the salt solution at pH 7.8. This process must be watched carefully and stopped before the naked cells, which retract around the explants, are washed away (all liberated fibroblasts appear to be damaged). Since digestion is never completed simultaneously in all cultures, the simplest procedure is to replace the digestion mixture in each culture with two drops of 50% plasma at the appropriate moment, to exhale alveolar air to depress the pH, and to stopper quickly. The liquid plasma should be rolled around the culture vessels several times and then be allowed to cover the explants and establish a bond with the wet glass before the third drop of medium or coagulant is added to induce simultaneous coagulation in all cultures thus accumulated.

juice 4%) is prepared in salt solution lacking both Ca and P, it contains approximately one half the usual levels of these elements. Though this solution delays the darkening of the central portion of transplants, there is no deficit in calcium compounds, and the central transplants eventually become opaque to transmitted light. This solution is superior to the usual formula for prolonged maintenance of cells in a plasma base.

Discussion. The demonstration of a 5-fold increase of Ca ions in a plasma base following serial renewals of a supernatant fluid, and of the necessity of hydrogen ions for its replacement, indicates that the plasma substrate in cell cultures is the site of an ionic exchange. A more complete analysis could be expected to show that not only Ca but also other preferentially adsorbed ions tend to replace less tightly bound but physiologically important ions. It may be inferred that cells maintained in an original or lightly patched plasma base spend a considerable portion of their existence in an inorganic environment quite different from what is intended and that the inorganic composition of renewal supernates might be improved by altering radically the ionic balance now normally present in fluids prepared from complex nutrients. It is also evident that existing data on the role of different ions in plasma cultures may require re-evaluation by culture methods which expose the cells directly to the fluids being studied.

The present data would appear to confirm the observations of Simms and Stillman[2] on the nature of at least one of the inhibitors which occur in plasma and adult tissues and is capable of being removed by tryptic digestion.

Summary. The adsorption of calcium phosphate complexes to plasma substrates, and the conditions required for their removal, indicate that the plasma gel is the site of an ionic exchange in which preferentially adsorbed ions may greatly alter the desired ionic balance. This process is one of the factors which causes plasma to become growth inhibitory during prolonged maintenance of cells in this type of substrate.

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17176. Tolerance of Normal and Partially Depancreatized Rats for Insulin.

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It was shown by this study that the average lethal dose of insulin is smaller in force-fed depancreatized rats than in normal rats.

Methods. Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets prior to the beginning of the experiments. At a weight of 310-320 g, some of the animals were subjected to a single-stage pancreatectomy[1] in which all but tiny remnants lying between the duodenum and bile duct were removed. After the rats recovered from the operation, they were placed in metabolism cages and were adapted to the force-feeding[2] of a medium carbohydrate diet (Table I) in amounts of 13 cc each morning and late afternoon. The urines were collected at 8:00 to 8:30 A.M. each day and were preserved by thymol. Urinary glucose was determined by the method of Benedict.[3]

Experiments and results. Severely diabetic rats were selected for these experiments. The average amount of glucose excreted by the 18 depancreatized rats used in this study was 6096 mg per day. The amount of glucose es-

[1] Ingle, D. J., and Griffiths, J. Q., Jr., Chapter 16, The rat in laboratory investigation. J. B. Lippincott Co., Philadelphia, 1942.

[2] Reinecke, R. M., Ball, H. A., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 44.

[3] Benedict, S. R., *J.A.M.A.*, 1911, **57**, 1193.